



1 of 3

PATENT
1209-0121P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicants: Ulf LANDEGREN Conf: 7960
Serial No.: 08/981,310 Group: 1641
Filed: December 16, 1997 Examiner: V. Portner
Re: ULTRASENSITIVE IMMUNOASSAYS

BRIEF ON BEHALF OF APPELLANTS

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Applicants:	Ulf LANDEGRENN	Conf:	7960
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BRIEF ON BEHALF OF APPELLANTS

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BRIEF ON BEHALF OF APPELLANTS

Assistant Commissioner for Patents
Washington, DC 20231

November 5, 2002

Sir:

The present Appeal Brief is respectfully submitted in connection with the above-identified application.

(1) REAL PARTY IN INTEREST

The Real Party in Interest of the present application is the inventor, Ulf LANDEGRENN.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences. However, Appellants have separately petitioned the refusal to enter the amendments to the specification submitted on May 15, 2002 for
11/05/2002 SZEWDIE1 00000039 08981310

01 FC:2252 200.00 CP

11/05/2002 SZEWDIE1 00000039 08981310

02 FC:2402 160.00 CP

reasons of new matter. Attached hereto, as Appendix II, is a copy of the petition.

(3) STATUS OF THE CLAIMS

Claims 1-6 and 8-10 are pending in the application and stand rejected per the Advisory Action of June 19, 2002 and Final Office Action of January 16, 2002. Claim 7 has been canceled. Claims 1-6 and 8-10 stand appealed.

(4) STATUS OF THE AMENDMENTS

The response to the Office Action filed on May 16, 2002 has not been entered on the basis that the amendments to the specification would "raise new issues with respect to New Matter." Appellants have separately petitioned the refusal to enter the amendments to the specification on the basis of new matter.

(5) SUMMARY OF THE INVENTION

The present invention is mostly broadly drawn to a test kit, which contains

- a) a first immobilized reagent having affinity to a

specific macromolecule, and

b) a second and a third affinity reagent specific for different determinants of the macromolecule, and modified with conjugatable oligonucleotides which conjugate through

i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;

ii) hybridization of the conjugatable oligonucleotides to each other; or

iii) ligation of the oligonucleotides,
wherein a signal is generated by nucleic acid amplification only when the second and third affinity reagents are closely bound on the target macromolecule, wherein the macromolecule is a protein.

The present invention is further drawn to an immunoassay which entails the steps of:

a) contacting a sample a first antibody linked to a solid support, wherein first antibody is specific for a first epitope on the antigen;

b) washing off excess sample;

c) incubating with a solution of a second and a third antibody specific for a second and a third epitope of the

antigen, and modified with conjugatable oligonucleotides, wherein the oligonucleotides conjugate to each other when said second and third antibody are both bound to the antigen through

- i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;
- ii) hybridization of the conjugatable oligonucleotides to each other; or
- iii) ligation of the oligonucleotides;

- d) washing off excess solution;
- e) amplifying the conjugated oligonucleotides; and
- f) detecting the amplified products.

(6) ISSUES FOR CONSIDERATION

(1) *Whether the specification contains support for claims 1, 3 and 4, which encompass a genus of kits that comprises three nucleic acid reagents or three co-factor reagents for determination of a protein, for the purposes of 35 U.S.C. §112, first paragraph.*

(2) Whether claims 1, 3 and 4 sufficiently define the invention with regard to nucleic acids or cofactors that bind to the protein macromolecule component of the claims, for purposes of 35 U.S.C. §112, second paragraph.

(3) Whether the invention of claims 1, 3, 4 and 5 are anticipated by Landegren et al. (U.S. Pat. No. 4,988,617).

(4) Whether the invention of claims 6 and 8-10 are obvious over the disclosures of Cantor et al. (U.S. Pat. No. 5,635,602) combined with Suzuki et al. and whether the invention of claims 1-6 and 8-10 are obvious over Cantor et al. combined with de la Monte et al. (U.S. Pat. No. 5,830,670).

(7) GROUPING OF THE CLAIMS

The claims on appeal do not stand together. The claims may be divided into the following separately patentable groups.

Group I - Claims 1-5

Group II - Claims 6 and 8-10

(8) ARGUMENTS ON THE ISSUES

(1) Whether the specification contains support for claims 1, 3 and 4, which encompass a genus of kits that comprises three nucleic acid reagents or three co-factor reagents for determination of a protein, for the purposes of 35 U.S.C. §112, first paragraph.

Claims 1, 3 and 4 have been rejected under 35 U.S.C. §112, first paragraph with the assertion that the specification does not provide support for a genus encompassing kits for detecting a protein macromolecule comprising either three nucleic acids or three cofactors as the first through third affinity reagents.

The rejection appears to be based on the position that the specification does not recite the specific combination that the binding affinity reagents are three nucleic acids or three cofactors and that the target macromolecule is a protein. Applicants note that claim 1 does not recite the specific species of a protein macromolecule and nucleic acid or cofactor binding reagents. Rather claim 1 recites affinity reagents that specifically bind to different determinants and that the macromolecule is a protein. Thus, claim 1 does not explicitly recite the combinations of elements in question, but rather a

genus that includes those combinations.

Dependent claim 3 recites that the affinity reagents may be nucleic acids or cofactors. The Examiner asserts that because claim 1 did not originally define the macromolecule as a protein there is no support for an assay for detecting a protein macromolecule with nucleic acids or cofactors as affinity reagents.

However, it has been well established that "it is not necessary that the claimed subject matter be described in *ipsis verbis* to satisfy the written description requirement. Nor does the statute require a specific example." Heymes v. Takaya 6 USPQ2d 1448, (Bd. Pat. App. & Int. 1988).

In the response of May 16, 2002, Applicants pointed to original claim 3 of the specification for support recited genus of claim 1.

Originally filed claim 3 recites, "wherein the affinity reagents are lectins, receptors, single chain antibodies, cofactors and nucleic acids." Thus, original claim 3 provides support for a genus encompassing kits comprising three nucleic acids or three cofactors. The specification was amended on page 3 to incorporate the subject matter of claim 3 into the

specification. The Examiner refused to enter the amendment to the specification on the grounds that it would be new matter. Applicants have separately petitioned this action by the Examiner. A copy of the petition is attached hereto as Appendix II.

The Examiner further asserted that the recitation in claim 3 is not sufficient because the specific combination of these affinity reagents with a protein macromolecule was not specifically disclosed. However, the only examples of macromolecules provided in the specification are proteins. Page 3, 3rd paragraph specifically mentions a protein as the macromolecule and specific examples of macromolecules of interest are provided on page 6, 4th paragraph, wherein myoglobin and growth hormone, both of which are proteins, are mentioned. In order to comply with the written description requirement, the specification "need not describe the claimed subject matter in exactly the same terms as used in the claims; it must simply indicate to persons skilled in the art that as of the [filing] date the applicant had invented what is now claimed."

Eiselstein v. Frank, 52 F.3d 1035, 1038, 34 USPQ2d 1467, 1470 (Fed. Cir. 1995) (citing Vas-Cath, 935 F.2d at 1562, 19 USPQ2d

at 1115, and In re Wertheim, 541 F.2d 257, 265, 191 USPQ 90, 98 (CCPA 1976)). In addition, "the failure of the specification to specifically mention a limitation that later appears in the claims is not a fatal one when one skilled in the art would recognize upon reading the specification that the new language reflects what the specification shows has been invented." All Dental Prodx, LLC v Advantage Dental Products, Inc. No. 02-1107 (Fed. Cir. Decided October 25, 2002). Given the disclosure of proteins as the only exemplified macromolecule in the specification and the specific recitation in original claim 3 that the affinity reagents may be cofactors or nucleic acids, one skilled in the art would conclude that claim 1, wherein the macromolecule is a protein and claim 3 which recites the use of nucleic acids or cofactors as affinity reagents are fully supported and meet the requirements under 35 U.S.C. §112, first paragraph. Claim 4, which depends from Claims 1-3 is also therefore necessarily supported.

(2) Whether claims 1, 3 and 4 sufficiently define the invention with regard to nucleic acids or cofactors that bind to

the protein macromolecule component of the claims, for purposes of 35 U.S.C. §112, second paragraph.

Claims 1, 3 and 4 have also been rejected under 35 U.S.C. §112, second paragraph as being indefinite. More specifically, the Examiner asserts that it is not clear what three nucleic acids or cofactors might bind to the same protein at the same time. However, the purpose of the requirements of 35 U.S.C. §112, second paragraph "is to ensure that the scope of the claims is clear so the public is informed of the boundaries of what constitutes infringement of the patent." M.P.E.P. §2173. "The primary purpose of the definiteness requirement is to ensure that the claims are written in such a way that they give notice to the public of the extent of the legal protection afforded by the patent, so that interested members of the public, e.g., competitors of the patent owner, can determine whether or not they infringe." All Dental Prodx.

Claim 1 clearly defines the boundaries of the invention as requiring three affinity reagents each of which binds to a different site on a protein macromolecule. "Interested members of the public, e.g., competitors of the patent owner, can determine whether or not they infringe" would readily be able to

determine whether a particular test kit falls within the scope of the claims and would infringe the patent by the simple test of whether there are three affinity reagents present, each of which binds to a different site on the macromolecule being assayed. In addition, with regard to the rejection by the Examiner regarding nucleic acid and co-factor affinity reagents specifically, it is clearly well-known in the art what nucleic acids and co-factors are. As such, one skilled in the art would readily recognize an affinity reagent as being a nucleic acid or cofactor, and the requirements for 35 U.S.C. §112, second paragraph, are fully met.

(3) Whether the invention of claims 1, 3, 4 and 5 are anticipated by Landegren et al. (U.S. Pat. No. 4,988,617).

Claims 1, 3, 4 and 5 under 35 U.S.C. §102(b) have been rejected as being anticipated by Landegren et al. (U.S. Pat. No. 4,988,617).

In the Office Action of January 16, 2002, the Examiner asserts that the claims encompass affinity reagents that bind to a macromolecule, which is a nucleic acid. However, claim 1 very clearly recites that the immobilized affinity reagent has

"affinity to a specific macromolecule"; that the second and third affinity reagents are "specific for different determinants of said macromolecule"; and that the macromolecule is "a protein." Thus, the assay kit of claim 1 would only detect a protein and assays and kits that would detect nucleic acids are clearly excluded from the claims.

To anticipate a claim, a prior art reference must disclose every feature of the claimed invention, either explicitly or inherently. See *Glaxo Inc. v. Novopharm Ltd.*, 52 F.3d 1043, 1047, 34 USPQ2d 1565, 1567 (Fed. Cir. 1995). Hazani v. I.T.C. 44 USPQ2d 1358, 1361 (Fed. Cir. 1997)

The assay method of Landegren '617 is "a method of detecting a nucleic acid change in nucleic acids." See the Title and Abstract. Thus, the only "macromolecule" in the assay of Landegren '617 is nucleic acid. As discussed above, the present invention of claims 1 and 3-5 is specifically drawn to a kit having affinity reagents that specifically bind to determinants on a protein macromolecule. The affinity reagents of Landegren et al. '617 are nucleic acids, which specifically bind to other nucleic acids. Thus, the affinity reagents of Landegren et al. '617 do not specifically recognize a protein macromolecule. As such, Landegren et al. '671 does not teach or suggest every feature of the claimed invention and the present invention is

not anticipated by Landegren et al. '617.

The present invention further differs from the disclosure of Landegren et al. because the second and third affinity reagents of the present invention have two separate parts, a first part that specifically binds to the analyte and a second part that is an oligonucleotide that modifies the first part and that is conjugatable.

With Landegren, on the other hand, the affinity reagents have only one part that is an oligonucleotide that binds to nearby portions on the target nucleic acid molecule.

Finally, the present invention differs from Landegren because whereas the second and third affinity reagents of the present invention are closely bound to the analyte to allow the nucleic acid portion of the affinity reagents to conjugate, with Landegren the nucleic acid probes bind to adjacent determinants.

Thus, the present invention is not anticipated by Landegren and withdrawal of the rejection is, therefore, respectfully requested.

(4) Whether the invention of claims 6 and 8-10 are obvious over the disclosures of Cantor et al. (U.S. Pat. No. 5,635,602)

combined with Suzuki et al. and whether the invention of claims 1-6 and 8-10 are obvious over Cantor et al. combined with de la Monte et al. (U.S. Pat. No. 5,830,670).

Claims 6 and 8-10 have been designated under the Grouping of the Claims as being separately patentable from claims 1-5. Claims 6 and 8-10 are specifically drawn to an immunoassay wherein the first through third affinity reagents are antibodies that specifically recognize different determinants on the target macromolecule. As discussed above, Landegren et al. is drawn to "a method of detecting a nucleic acid change in nucleic acids" using nucleic acids. There is no motivation to modify the assay of Landegren et al. so as a) to be able to detect proteins rather than nucleic acids or b) to replace the single component nucleic acid probes with two-component affinity reagents made specifically from an antibody linked to a conjugatable nucleic acid, wherein the oligonucleotides on the antibody affinity reagents only conjugate to each other when said second and third antibody are both bound to the antigen. As such, the invention of claims 6 and 8-10 is separately patentable from that of claims 1-5.

Claims 6 and 8-10 have been rejected under 35 U.S.C. §103 as being over Cantor et al. (U.S. Pat. No. 5,635,602) combined with Suzuki et al. and of claims 1-6 and 8-10 as being obvious over Cantor et al. combined with de la Monte et al. (U.S. Pat. No. 5,830,670). Both of these rejections will be addressed as one issue, as the arguments are applicable to both rejections because the deficiencies in Cantor et al. are not compensated for by either Suzuki et al. or de la Monte et al.

Cantor et al. discloses an assay where two reagents with affinity for a target protein have already been connected via double stranded DNA sequences when they are added to a sample. With the present invention, the second and third affinity reagents each are connected to a single DNA strand and are added as separate reagents to the immobilized target molecule. With the assay of the present invention, the conjugation of the second and third affinity reagents after binding indicates the juxtaposition of the two target determinants.

As a result of the second and third affinity reagents being completely dissociated upon addition, with the invention, there is almost no background signal because most non-specific binding events will not result in the generation of a signal. At the

same time, specific signal can be amplified. These advantages are not possible with the assay of Cantor et al. because with Cantor et al. once one of the affinity probes becomes immobilized there is an increased risk that the connected probe will also become immobilized even if there is no correct target determinant, increasing the background signal. In addition, with Cantor et al. before the oligonucleotides can be amplified, all the connected nucleic acids must first be separated using restriction digestion of the oligonucleotide duplex. Any non-specifically bound complex that is not cleaved will serve as a template for amplification, further creating non-specific background signal.

The Examiner asserts that the method of Cantor et al. will result in reduced non-specific binding. However, as discussed above, by adding the second and third affinity reagents as separate, dissociated reagents, the present invention can reduce non-specific binding. The reduction in non-specific binding associated with the present invention is not possible with the assay of Cantor et al. because with Cantor et al. affinity reagents are joined together prior to addition to the assay. The disadvantages associated with Cantor et al. significantly

reduce the assay sensitivity and problems associated with cleaving the connecting oligonucleotide duplex are discussed in column 13, line 51 of Cantor et al.

Suzuki et al. disclose a form of an "immuno PCR" procedure that is disclosed in Sano et al. (1992) Science 258: 120-122. Both Suzuki et al. and the original Sano et al. publication describe that the binding of an antibody to an analyte may be detected using amplification of a nucleic acid sequence added to the antibody bound to the immobilized analyte. With the present invention, however, two separate affinity reagents are required to bind to the immobilized analyte and only when the two affinity reagents are bound to the analyte can the oligonucleotides be conjugated and a signal generated. As noted above, the assay of the present invention reduces almost all the background signal created by non-specific binding by requiring that both the second and third affinity reagents specifically bind before signal is generated. This advantage is not possible with Suzuki et al., nor is this advantage possible with Cantor et al.

As evidence of the unexpected results associated with the present invention attached hereto as Appendix III is a journal

article of Fredriksson et al. *Nature Biotechnology*, Vol. 20: 473-477 (2002), (the present inventor is the final, i.e. primary author of the article). The data of Figure 6 compares the present assay procedure, which is dependent on the conjugation by ligation of two nearby oligonucleotides located on one analyte binder, to the assay format encompassed by Suzuki et al. wherein any bound antibody may be amplified. The data demonstrates the advantages associated with the present invention, which are in no way suggested by either Suzuki et al. or Cantor et al.

The Examiner has asserted that the data of Figure 6 is not probative because the Figure was not in the originally filed specification. However, under M.P.E.P. §706.02(g) publications may be relied on as evidence of unexpected results. The Examiner also raises the issue that the data of Figure 6 was obtained using specific reagents and that the claims are not limited to these reagents. However the unexpected improved properties associated with the present invention result from the use of three affinity reagents that all bind to different sites, wherein two of the affinity reagents have been modified with conjugatable oligonucleotides that will only conjugate to one another after

the affinity reagents bind to the target protein. The unexpected properties demonstrated in Figure 6 of the Fredricksson et al. publication, would be expected by one skilled in the art to be observed with any combination of affinity reagents used, provided that the affinity reagents bind to three independent sites on the target protein. Thus, the data of Figure 6 is fully commensurate with the scope of the invention and demonstrates the unexpected properties associated with the claimed invention; and it is not possible to achieve the present invention and the advantages achieved the invention by combining Suzuki et al. with Cantor et al.

It is further not possible to achieve the present invention by combining de la Monte et al. with Cantor et al. De la Monte et al. does not disclose a new procedure for detecting proteins. The disclosure of de la Monte et al. is directed to a particular class of proteins associated with Alzheimer's disease. The assays disclosed in de la Monte et al. are conventional sandwich ELISA assays for detecting the Alzheimer's proteins. De la Monte et al. disclose in claim 4, the use of three antibodies, however no nucleic acid conjugation or amplification is disclosed. In claim 6 of de la Monte et al. PCR analysis is

disclosed. However, it is evident from column 6, lines 37-43, PCR amplification is contemplated for the detection of only single antibody binding. As such, the procedures disclosed in de la Monte et al. would also have the same background problems discussed above regarding Suzuki et al. and Cantor et al. There is no suggestion in de la Monte et al. of requiring the conjugation between oligonucleotides associated with closely bound probes. Nor is there any suggestion in de la Monte of the unexpected improvements of reduced background and increased sensitivity associated with the present invention. As such, it is not possible to achieve the present invention by combining de la Monte et al. with Cantor et al. and withdrawal of the rejection is respectfully requested.

The Examiner asserts that Suzuki et al. suggests modifying immunoPCR assays with an immobilized antibody to improve results and that de la Monte et al. teach the use of an immobilized antibody and immunoPCR in disease diagnosis. As discussed above, the improved sensitivity and reduced back ground associated with the present invention results from the use of three independent affinity reagents that bind to separate sites

on the target protein, wherein two of the affinity reagents have been modified with conjugatable oligonucleotides that will only conjugate to one another after the affinity reagents bind to the target protein. None of the references teach the premise for the invention regarding the conjugation of the nucleic acids on the second and third affinity reagents after binding. As such, the invention is neither disclosed nor suggested by the references and cannot be achieved if the references are combined in their teachings. Withdrawal of the rejections is therefore respectfully requested.

If necessary, the Commissioner is hereby authorized in this, concurrent, and further replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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Appendices: I - Claims on Appeal
II - Petition Under 37 C.F.R. §1.181 and M.P.E.P. §§2163.06
III - Fredriksson et al. Nature Biotechnology, Vol. 20:
473-477 (2002)

CLAIMS ON APPEAL

1. (Four times Amended) A test kit comprising

a) a first immobilized reagent having affinity to a specific macromolecule, and

b) a second and a third affinity reagent specific for different determinants of said macromolecule, and modified with conjugatable oligonucleotides which conjugate through

i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;

ii) hybridization of the conjugatable oligonucleotides to each other; or

iii) ligation of the oligonucleotides,
wherein a signal is generated by nucleic acid amplification only when said second and third affinity reagents are closely bound on said macromolecule; wherein said macromolecule is a protein.

2. (Amended) The test kit according to claim 1, wherein the affinity reagents are antibodies, and the macromolecule is a specific antigen.

3. (Amended) The test kit according to claim 1, wherein the affinity reagents are lectins, receptors, single chain antibodies, cofactors or nucleic acids.

4. (Amended) The test kit according to any one of the claims 1-3, wherein the oligonucleotides are complementary to each other.

5. (Amended) The test kit according to claim 1, further comprising a ligase.

6. (Thrice Amended) An immunoassay for detection of a specific antigen, comprising:

a) contacting a sample suspected of containing said specific antigen with a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen;

b) washing off excess sample;

c) incubating with a solution of a second and a third antibody specific for a second and a third epitope of said antigen, and modified with conjugatable oligonucleotides,

wherein said oligonucleotides conjugate to each other when said second and third antibody are both bound to said antigen through

- i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;
- ii) hybridization of the conjugatable oligonucleotides to each other; or
- iii) ligation of the oligonucleotides;

- d) washing off excess solution;
- e) amplifying said conjugated oligonucleotides; and
- f) detecting the amplified products.

8. (Thrice Amended) An immunoassay according to claim 6, wherein the conjugation occurs through hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides.

9. (Twice Amended) An immunoassay according to claim 6, wherein the conjugation occurs through hybridization of the conjugatable oligonucleotides to each other.

10. (Amended) An immunoassay according to claim 8, wherein
the conjugation occurs through ligation of the oligonucleotides.

Protein detection using proximity-dependent DNA ligation assays

Simon Fredriksson¹, Mats Gullberg¹, Jonas Jarvius¹, Charlotta Olsson¹, Kristian Pietras², Sigrún Margrét Guðstafsdóttir¹, Arne Östman², and Ulf Landegren^{1*}

The advent of *in vitro* DNA amplification has enabled rapid acquisition of genomic information. We present here an analogous technique for protein detection, in which the coordinated and proximal binding of a target protein by two DNA aptamers promotes ligation of oligonucleotides linked to each aptamer affinity probe. The ligation of two such proximity probes gives rise to an amplifiable DNA sequence that reflects the identity and amount of the target protein. This proximity ligation assay detects zeptomole (40×10^{-21} mol) amounts of the cytokine platelet-derived growth factor (PDGF) without washes or separations, and the mechanism can be generalized to other forms of protein analysis.



Sensitive and specific methods are required to study concentration, location and colocalization, and secondary modification of the large sets of proteins that are predicted from genome sequence data—stringent demands that are difficult to meet with current techniques. By contrast, methods in use for detection of specific nucleic acid sequences, such as PCR, offer excellent sensitivity and specificity¹. This is only partially a consequence of the strong and predictable affinity between complementary nucleic acid strands. Of greater importance is the requirement that pairs of probes recognize a given target sequence, and that only this dual interaction can initiate an amplified detection signal. Such a requirement for dual and proximate recognition is also a frequent element of cellular recognition reactions in signal transduction and gene regulation².

We have developed an analogous method for *in vitro* analysis of proteins and other macromolecules, termed proximity ligation. The method depends on the simultaneous and proximate recognition of target molecules by pairs of affinity probes, giving rise to an amplifiable detection signal. The molecule we targeted was the homodimer of the platelet-derived growth factor B-chain (PDGF-BB), a cytokine with growth- and differentiation-promoting effects³. As affinity probes we used so-called DNA aptamers, a class of oligonucleotide reagents obtained through a process of *in vitro* selection for affinity to a target molecule^{4,5}, and shown to be suitable for analytical applications^{6,7}. A DNA aptamer with affinity for PDGF-BB (ref. 8) was extended by additional sequence elements at either the 5' or the 3' end, forming a proximity probe pair. When pairs of probes bind PDGF-BB, the free ends of their sequence extensions are brought sufficiently close to hybridize together to a subsequently added connector oligonucleotide, allowing the ends to be joined by enzymatic DNA ligation. The detected protein molecules thus promote the ligation reaction by bringing together the free ends of the proximity probe pairs. The ligation products can then be replicated by nucleic acid amplification through PCR, while unreacted probes remain silent (Fig. 1).

Results

Detection of PDGF-BB by proximity ligation. On the basis of crude molecular modeling, we selected DNA extensions of the affinity probes of around 40 nucleotides each. However, we found that the length of the extensions could be varied over a considerable range with negligible effects on ligation efficiency and nonspecific signal (data not shown). The method thus also has the potential to allow analysis of large proteins.

The connector oligonucleotide was designed to base-pair to ten nucleotides at the free end of each member of the proximity probe pair. A short hybridizing length and 5' and 3' end sequences that do not base-pair to the proximity probes were selected to prevent the connector oligonucleotide from giving rise to ligation-independent amplification products by acting as a primer and/or template for amplification. Longer connector oligonucleotides of 14 + 14 or 16 + 16 hybridizing nucleotides yielded false PCR products (data not shown). The connector oligonucleotide was added in large molar excess over proximity probes to ensure efficient formation of ligation substrates.

We carried out homogeneous assays for PDGF-BB, requiring no washes or separations, by first preincubating proximity probes with the samples at room temperature for 1 h or at 37°C for 15 min. We then added a mixture that included all reagents required for both probe ligation and quantitative PCR with real-time detection⁹, thereby also diluting the reaction. Finally, we transferred the reactions to a fluorometric PCR instrument for detection of ligated molecules.

To optimize assay parameters and investigate reaction mechanisms, we preincubated a proximity probe pair with a fixed amount of PDGF-BB or with no PDGF-BB. Then we added variable amounts of connector oligonucleotide and allowed it to act as template for the ligation of proximity probe pairs for 5, 25, or 125 min before PCR with real-time detection (Fig. 2A). With addition of high concentrations of connector oligonucleotide, colocalized ends of proximity probes were joined rapidly and extended incubation resulted in little increase in either signal or background. This lack of further ligation at high connector concentrations renders the assay quite robust and

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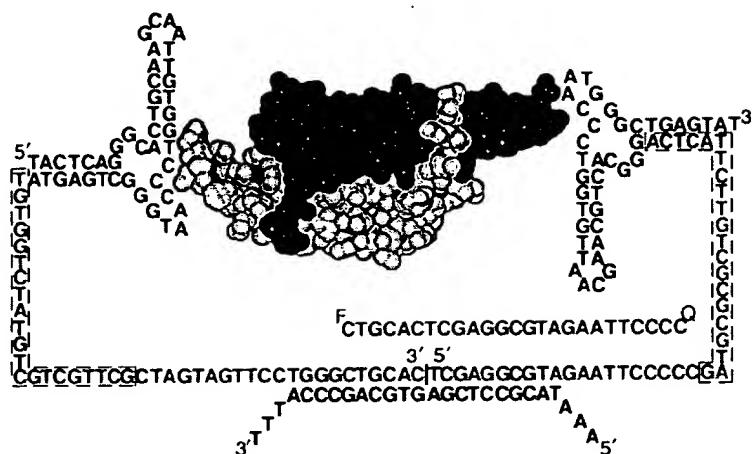


Figure 1. Schematic view of the homodimeric PDGF-BB (ref. 23) bound by two aptamer-based proximity probes, A1 and A2, for detection by proximity ligation. The sequence of the aptamer 41t specific for the PDGF B-chain⁸ is shown in black, sequence extensions to be joined by ligation upon hybridization to a common connector oligonucleotide are shown in blue (A1) and red (A2), and primer sites for PCR are boxed. A probe for real-time detection of PCR products via the 5'-nuclease assay is shown in green with a 5' FAM fluorophore and a 3' TAMRA quencher.

probably reflects the hybridization of nearly all free proximity probes to one connector oligonucleotide each, inhibiting the formation of new ligation substrates. A connector oligonucleotide concentration of 400 nM was selected for subsequent experiments, and ligation was allowed to proceed for 5 min before amplification.

Next, we varied preincubation volumes and concentrations of proximity probes. All samples were then brought to a final volume of 50 μ l by adding the ligation and amplification mix (Fig. 2B). Increased detection sensitivity resulted if PDGF-BB-independent ligation events were limited by using a minimal amount of probes, and by preincubating the proximity probes and sample in the smallest possible volume to promote binding. In further experiments we used 20 pM proximity probes in a preincubation volume of 5 μ l. The very low probe concentration during the ligation phase of the assay keeps target-independent ligation events to a minimum, and requires a mere 100 attomoles of proximity probes per assay.

By assuming that the ends of oligonucleotide pairs that have bound a protein molecule are constrained within a sphere of a radius defined by the size of the protein (~60 Å) and taking into account the maximal length of the nucleotide extensions (5 Å per nucleotide), we estimate that under these experimental conditions local concentration of ligatable ends of the proximity probes increases by a factor of at least 10^6 upon target binding.

As few as 24,000 PDGF-BB molecules were reproducibly detected over background plus two standard deviations by proximity ligation, ~1,000-fold fewer than were detected by a sandwich enzyme-linked immunosorbent assay (ELISA) for the same protein (Fig. 3). The linear range for quantitation extended over a >1,000-fold concentration range, with sufficient precision to distinguish 2-fold dilutions of the protein. The error associated with the analysis is of the same order as that of quantitative PCR with real-time detection alone, calculated for the number of amplicons generated: 35% coefficient of variation (CV) for

proximity ligation (averaged in Fig. 3) and 32% CV for 88 replicates of the diluted amplicon analyzed by real-time PCR.

Under the reported conditions, approximately 1 PDGF molecule in 25 had bound 2 different proximity probes that were subsequently joined by ligation. The reason that not all protein molecules trigger a ligation reaction is probably a consequence of several factors: most importantly, at 20 pM concentrations of proximity probe, well below the reported K_d for the aptamers (~100 pM), only a minority of proteins are expected to have bound one proximity probe, and even fewer will have bound two. Moreover, only one in two such complexes of target protein with two proximity probes involve probe pairs with one 5' end and one 3' end available for ligation. Finally, the ligation efficiency of juxtaposed proximity probe ends could be <100% as a result of imperfections of oligonucleotide synthesis.

The aptamers have a reported 500-fold lower affinity for the PDGF A-chain compared with the B-chain, and accordingly the PDGF-AA homodimer was not detected in the assay and neither were the PDGF-BB homologs PDGF-CC (ref. 10) and PDGF-DD (refs 11,12) (Fig. 3). Furthermore, the PDGF-AB heterodimer was not detected, demonstrating that the assay is truly proximity dependent. The detection reaction could be specifically inhibited through the addition of a fragment of the PDGF β -receptor¹³, known to compete with the aptamer for binding to PDGF-BB (ref. 8). PDGF-BB was also analyzed in the presence of fetal calf serum (FCS), cerebrospinal fluid (CSF), and Eagle's minimal essential medium (EMEM) to demonstrate the applicability of the assay in realistic biological samples and cell culture medium (Fig. 4). Closely similar results were obtained in biological samples and in defined media (compare Figs 3 and 4).

The concentration of PDGF-BB in a human serum sample was demonstrated by homogeneous proximity ligation to be 0.2 nM (6 ng/ml), compared with a value of 0.13 nM as determined by ELISA. By contrast, the PDGF-BB content in the corresponding plasma sample was <1 pM as determined by ELISA and <0.2 pM using proximity ligation. The proximity ligation assay further revealed a concentration of 1 pM PDGF-BB in conditioned medium from a 36 h culture of the human anaplastic thyroid carcinoma cell line SW-1736 (ref. 14), shown by northern blot analysis to

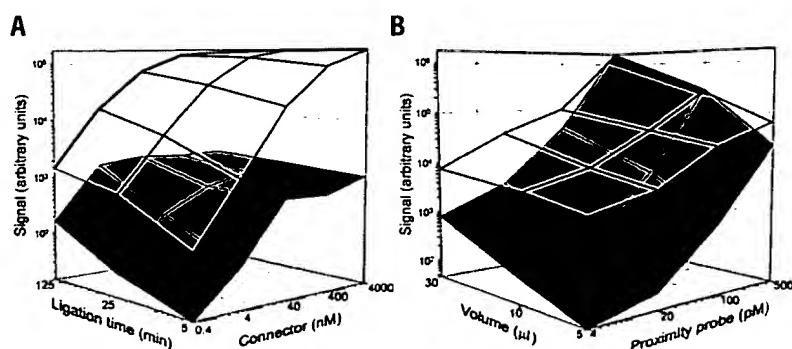


Figure 2. Optimization of reaction conditions for the proximity ligation assay. (A) Effect of connector oligonucleotide concentration and time of ligation on signal and background. (B) Influence of preincubation volume and probe concentration. The upper, yellow layers connect the signals recorded in reactions with 5.6 attomoles of PDGF-BB, while the lower, red layers represent the target-independent background, expressed in arbitrary units. Averages of duplicate measurements are shown.

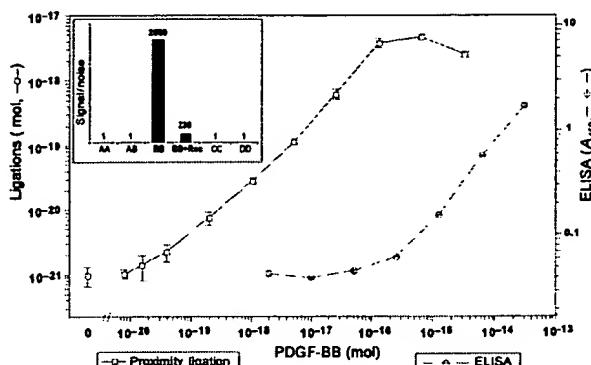


Figure 3. Detection of a dilution series of PDGF-BB by proximity ligation (solid line) and by sandwich ELISA (dashed line). Average values of triplicate and duplicate determinations, respectively, are shown with s.e.m. In the inset panel, results are shown of analyses of 3 fmol PDGF-AA, -AB, -BB, -BB with 1.5 pmol of the PDGF β -receptor, PDGF-CC, and -DD.

transcribe the PDGF-B gene (N. Heldin, personal communication). This corresponds to an estimated production of 1,500 cytokine molecules per cell per day.

Proximity ligation detection of human α -thrombin. We also designed proximity probes for detection of human α -thrombin. A pair of DNA aptamers binding two distinct sites^{15,16} were extended with sequences for ligation and amplification to form a proximity probe pair (Fig. 5). The sensitivity of detection illustrates that the proximity ligation strategy is also effective when using affinity probes for two distinct determinants on one protein, and that the procedure should also be useful for detecting interactions between different protein molecules.

Solid-phase proximity ligation. The homogeneous proximity ligation assay is simple to standardize and to carry out for large sets of samples. It eliminates the need for extensive, carefully controlled washes as required in solid-phase assays. However, for samples containing potential inhibitors of ligation or amplification, or in which the concentration of analyte is exceedingly low, solid-support immobilization of the protein can serve both to purify and to concentrate the samples. It also permits removal of unbound probes through washes before the ligation step, further reducing target-independent probe ligation. By immobilizing PDGF-BB through binding to antibody-coated reaction wells, we

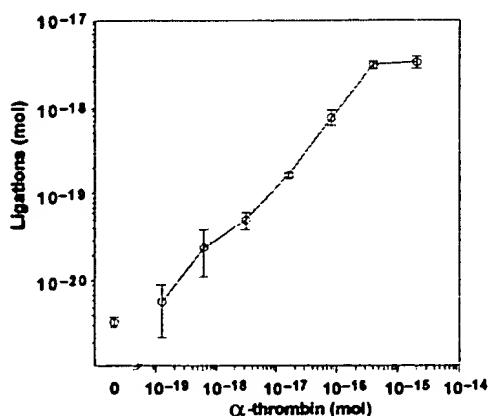


Figure 5. Detection of human α -thrombin by homogeneous proximity ligation. The x-axis indicates molar amounts of protein. Averages of duplicate measurements are shown with s.e.m.

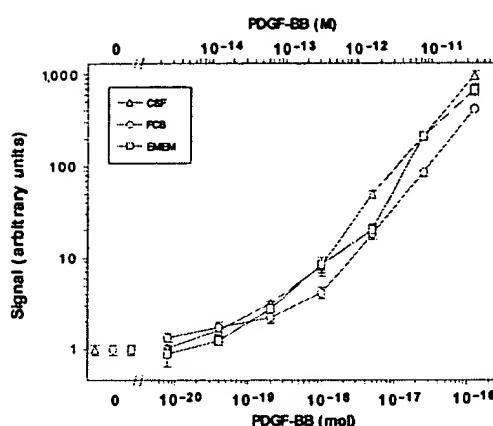


Figure 4. Homogeneous detection of PDGF-BB in the presence of complex biological fluids and cell culture media: FCS, EMEM, and human CSF. The top x-axis indicates concentration of PDGF-BB, and the corresponding molar amounts are shown at the bottom x-axis. Signals are normalized to a scale of arbitrary units of duplicate measurements with indicated s.e.m.

detected low-femtomolar concentrations of the protein using the same pair of proximity probes as was used in the homogeneous assay (Fig. 6). This compares with a low-picomolar detection threshold in the ELISA (Fig. 3).

PCR has previously been used in solid-phase protein assays for sensitive detection of single affinity probes labeled with DNA strands (immuno-PCR; ref. 17). To compare this strategy with proximity ligation, variable amounts of PDGF-BB were bound to antibodies immobilized in microtiter wells. This permitted us to compare detection of binding by a single aptamer having an oligonucleotide extension amplifiable by PCR, with the use of a proximity probe pair that could be amplified only upon ligation (Fig. 6). Across a range of probe concentrations and washing conditions, immuno-PCR resulted in a considerably higher background, and thereby a reduced sensitivity compared with proximity ligation. In the immuno-PCR approach, any nonspecifically bound probes can contribute to background signals, whereas proximity ligation requires proximity of pairs of probes to yield a signal.

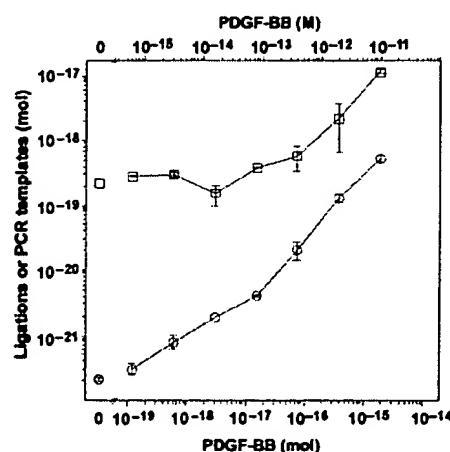


Figure 6. Dilution series of PDGF-BB analyzed in a solid-phase assay, either by proximity ligation (circles) or by aptamer-based immuno-PCR (squares). The top x-axis indicates concentration of PDGF-BB analyzed with the corresponding molar amounts at the bottom x-axis. Results are represented as averages of duplicate measurements with s.e.m.

Discussion

There are advantages to using aptamers in proximity ligation in that the attachment of DNA extensions is trivial, isolation of aptamers can be automated and scaled up¹⁸, and high-affinity reagents are regularly obtained, as demonstrated in a recent study of 100 protein-specific aptamers¹⁹. Moreover, this class of affinity probes can be shared in the research community by publishing the nucleotide sequence of appropriate reagents, permitting standardization of protein assays between labs. Nonetheless, most currently available reagents for protein binding are themselves proteins. Preliminary results demonstrate that monoclonal antibodies are suitable as proximity probes after coupling of oligonucleotides (M.G., unpublished results), and that heteroantisera, affinity probes prepared by phage/ribosome display techniques²⁰, or other types of affinity reagents should also be useful.

The proximity-dependent ligation assay converts the detection of any macromolecule for which suitable pairs of affinity probes exist into a more convenient reaction for detecting nucleic acids. The use of a DNA reporter for the detection reactions permits simultaneous analysis of large sets of proteins by encoding tag sequences in the amplified segment for separate detection, for example by hybridization to a standard oligonucleotide array. Proximity ligation should also enhance the sensitivity and specificity of localized *in situ* detection of proteins, and it could provide a valuable means to investigate secondary modifications of specific proteins, as well as the interaction of pairs of biomolecules in macromolecular complexes.

Experimental protocol

Detection of PDGF-BB by proximity ligation. Unless otherwise indicated, 1 µl aliquots, containing 5.6 attomoles PDGF-BB diluted in 137 mM NaCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4, 2.7 mM KCl, 1 mM MgCl₂, and 1% BSA, were added to optical tubes (Applied Biosystems (ABI), Foster City, CA) containing 20 pM of the proximity probes A1 and A2 in a total volume of 5 µl (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.3 mM MgCl₂, 0.1% BSA). Upon addition of the combined mix for ligation and amplification, the samples contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.4 units T4 DNA ligase (Amersham Biosciences, Uppsala, Sweden), 400 nM connector oligonucleotide, 80 µM ATP, carboxy-x-rhodamine (ROX) internal fluorescence standard, 0.2 mM dNTPs, 0.5 µM primers (forward: 5'-ATGTCCTC-TATCTCGTCTCG-3'; reverse: 5'-TGACTAAGAACAGCCCCCAT-3'), 50 nM probe for the 5' nuclease assay, and 1.5 units AmpliTaq Gold polymerase (ABI) in 50 µl. After a 5 min ligation reaction at room temperature, the reactions were transferred to the real-time PCR instrument for temperature cycling: 10 min at 95°C, and then 15 s at 95°C and 60 s at 60°C, repeated 45 times (ABI PRISM 7700). Signal-to-noise values represent the number of detected ligation products divided by the number of ligation products arising in a sample without PDGF-BB. The number of ligation products was calculated from a standard curve of diluted amplicons.

In experiments for which the concentration of connector oligonucleotide was varied, we took precautions to avoid an influence by the complementarity between the 5' nuclease probe and the connector oligonucleotide. After a preincubation with proximity probes in a 5 µl volume, a 35 µl ligation mix was added and the ligation reaction was terminated by heating at 95°C for 10 min, followed by the addition of reagents for PCR to a final volume of 50 µl. Furthermore, separate standard curves of diluted amplicons were prepared for each connector concentration.

PDGF-BB detection in biological samples. Before use, FCS was heated to 65°C for 20 min to inactivate the α2-macroglobulin that binds PDGF-BB,

blocking the binding of proximity probes²¹. Reactions (5 µl, with 3.4 µl sample and 20 pM proximity probes) were incubated at 37°C for 20 min with 16 µg/ml poly(A) oligonucleotide. In the PCR, MgCl₂ (3.5 mM) was used.

PDGF-BB quantitation in biological samples. PDGF-BB was quantified in serum and plasma by adding known amounts of PDGF-BB to a diluted sample and calculating the endogenous concentration from the increase in signal. The specificity of the assay was confirmed by blocking probe binding with an excess of soluble PDGF β-receptor, bringing the signal to background levels. The ELISA was done according to the supplier's instructions (R&D Systems, Minneapolis, MN). The incubation mix also contained 16 µg/ml poly(A) oligonucleotide and PCR was done with 3.5 mM MgCl₂.

PDGF-BB expression by the SW-1736 cell line, grown in 2 ml EMEM supplemented with 1% FCS, was assayed by homogeneous proximity ligation of 1 µl samples, diluted 5-fold in PBS with 0.1% BSA, and quantified by reference to analyses of known concentrations of the protein diluted in growth medium. A total of 300,000 cells were plated and after 36 h, 514,000 cells were counted. Specificity of protein detection was confirmed by blocking the signal with an excess of PDGF β-receptor.

Detection of thrombin by proximity ligation. Experimental procedures were identical to those for analysis of PDGF-BB except as indicated below. The proximity probes were Thr1 (5'-CAGTCCGTGTAGGGAGGTTCGGGT-GACTCTCGGAACTATCTAGCGCTGACTGACTGGGCATGCTACCAA-GAGG-3') and Thr2 (5'-P-GTCATCATTCGAATCTACTCCAATCCGTTATTAGGCTACTGACTACTGGTGGTGAGGTTGGGTAGTCACAAA-3'), aptamer sequences underlined. The connector oligonucleotide was 5'-AAGAATGATGACCCTCTTGCTAAAAA-3', and the 5' nuclease probe was 5'-TET TGTACCTGACTGGGCATCTACCAAGAGG-3'-TAMRA (TET, tetrachlorofluorescein; TAMRA, carboxytetramethylrhodamine). Samples were diluted in 100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer, pH 6.5, and 1% BSA, and preincubated with proximity probes Thr1 (15 pM) and Thr2 (20 pM) in 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂. The PCR buffer contained 1.9 mM MgCl₂. Primers for PCR were 5'-GTACTT-CGTGGAACTATCTAGCG-3' and 5'-AATACCGATTGCATACCCATT-3'.

Detection of PDGF-BB by solid-phase proximity ligation and immuno-PCR. An anti-PDGF antiserum immunoglobulin fraction²² (500 ng) in a 20 µl volume was added to optical tubes for real-time PCR, followed by blocking with 1% BSA. Up to 200 µl of sample was then incubated together with 5 nM of proximity probes or with 0.1 nM of immuno-PCR reagent. Unbound probes were removed by washes (200 µl) using a multichannel pipette (proximity ligation 8 times with 3 flushes per wash, immuno-PCR 8 times with 4 flushes per wash (washes 1 and 2, PBS with 0.02% SDS; washes 3-7, PBS; wash 8, H₂O)). A ligation mix (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.15 mM ATP, 50 nM connector oligonucleotide, 2 U T4 DNA ligase in 20 µl) was added and the reactions were kept at 30°C for 5 min and then at 80°C for 20 min. Next, a PCR mix was added to a final volume of 50 µl, containing ROX internal fluorescence standard, 0.1 mM dNTPs, 0.2 µM primers, 50 nM probe for the 5' nuclease assay, and 1 unit AmpliTaq Gold polymerase.

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